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ENVIRONMENTAL CONTROL OF AMYLASE PHENOTYPE IN AMPHIPODS OF THE GENUS *GAMMARUS*

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ABSTRACT

The amylases of three species of *Gammarus* amphipods, *G. palustris, G. mucronatus,* and *G. lawrencianus,* were studied using polyacrylamide gel electrophoresis. In all three species there are two principal zones of activity (*Amy-1* and *Amy-2*), but occasional individuals exhibit a third band (*Amy-X*). *Amy-X* variants are more common in the deeper water species and in collections made during late winter or early spring than in the shallower water species or in summer collections. Experimental work shows that *Amy-X* can be induced by a diet rich in amylose or glycogen, but not by amylopectin or other foods, and by reducing the temperature at which they are maintained. It is hypothesized that the relative expressions of *Amy-X* and *Amy-1* vary seasonally, and that this variation is an adaptive response to seasonal variation in diet, or conditions for the hydrolysis of substrate.

INTRODUCTION

One of the least understood phenomena in population biology is how the environment influences the genetic contents and phenotypic expressions of natural populations. Amphipods of the genus *Gammarus* are useful in the study of this question because they are distributed among a variety of aquatic environments, ranging from freshwater, through estuarine, to marine. *Gammarus* species found in the intertidal zone in marine and estuarine environments can be easily collected in large numbers and tend also to be hardy enough to culture in the laboratory. Thus, data on gene frequencies in natural populations can be obtained easily, and hypotheses relating to the effects of environmental factors on the population can be tested by laboratory experiment. Furthermore, the amphipods are an intrinsically interesting group for evolutionary studies because they are believed to be currently in the midst of an explosive adaptive radiation (Bousfield, 1973).

This paper reports work on amylase variation in populations of *Gammarus palustris, G. mucronatus,* and *G. lawrencianus.* These three species are estuarine to marine in distribution, co-occur at a number of localities in the New York area, and are zoned ecologically in areas of sympatry, from the high intertidal through the low intertidal-subtidal, in the order listed above. Amylase was chosen for study because the enzyme acts upon a heterogeneous substrate set which is externally derived. Environmental variation is expected to influence isozyme variation at such loci more than at loci coding for enzymes which act upon internally derived homogeneous substrate sets (Gillespie and Kojima, 1968; Kojima, *et al.,* 1970; Johnson, 1973, 1975)

Amylases in these species are coded by at least two distinct loci (*Amy-1* and *Amy-2*) and can easily be resolved by electrophoresis on polyacrylamide gels. In occasional individuals of all three species, a third band (*Amy-X*), running close to *Amy-1,* is

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clearly exhibited. This band was initially ignored, because of its unreliable appearance, until it was observed that collections made during colder weather yielded a higher proportion of *Amy-X* individuals than those made during warmer weather. This observation suggested that differential expression of amylase isozymes in *Gammarus* could be controlled by environmental variation. Therefore, a series of laboratory experiments was performed in order to identify environmental factors that might influence the expression of *Amy-X*. Temperature and diet were the two factors chosen.

Materials and Methods

All animals used in these experiments were collected at low tide in the intertidal zone at two sites in Brooklyn, New York. *Gammarus palustris* and *G. mucronatus* were taken at the Cross Bay Boulevard Bridge, (mean salinity, 2.4%) at the head of Jamaica Bay and *G. lawrencianus* was taken at Knapp Street, (mean salinity, 3.0%). Collections were made during 1977–1979. Experimental animals were maintained in the laboratory in filtered water from the collection sites on a 12:12 light dark cycle for times and at temperatures specified in Table I. Holding (acclimation) temperatures before the experiments were chosen to match collection temperatures.

The foods used in the feeding experiments were: *Ulva lactuca* or *Enteromorpha intestinalis* ("green algae"), *Punctaria sp* (= "brown algae"), commercially available shrimp meat, and four artificial foods: (1) agarose flakes (Indubiose A45, L'Industrie Biologique Francaise), (2) amylopectin (Sigma #A-8515)-agarose flakes (2 parts amylopectin to 1 part agarose), (3) amylose (Sigma #A-0512)-agarose flakes (2:1), and (4) mussel glycogen (Sigma #G1508)-agarose flakes (2:1). The flake foods were prepared by dissolving the constituents in boiling water, coating a glass plate with the solution, drying the gel, and scraping off the film.

Properties of the artifical foods

All four of the flake foods maintained their overall structure in water for the durations of the feeding experiments. Separate experiments were performed in order to determine whether the starches would leach out of the films into solution. Fifty milligram portions of the different films were added to 10 ml portions of distilled water and the mixtures were sampled after 1, 24, and 48 hours. The agarose film and its soak gave negative results when tested with Gram's iodine reagent. The amylose soak was iodine negative after 1 and 24 hours but gave a weak positive reaction after 48 hours. The amylopectin soak gave a strong reaction after only 1 hour, but the film retained some amylopectin and gave a moderate reaction even after 48 hours in water. The glycogen chosen gives only a weak reaction with iodine so it was not tested. Glycogens, however, are of low solubility in cold water. The amphipods fed avidly upon the three flake foods containing starch or glycogen. They also were observed to feed upon the agarose film, but much less of it was eaten.

Two clarifications of usage should be made here. First, the term "induction" is used in its broadest sense in this paper, to describe the phenomenon in which environmental factors lead to the production of a new enzyme form. Its usage is not meant to imply any particular molecular mechanism out of the several possibilities which are considered in the discussion. Second, the induction of *Amy-X* was followed experimentally in terms of the expression of *Amy-X relative* to that of *Amy-1*. That is, increases in the relative expression of *Amy-X* were ascribed solely to its induction, and the effect of "repression" of *Amy-1* was ignored. This convention, choosing *Amy-1* as the fixed reference, was adopted because absolute activities cannot easily be read

from amylase zymograms, and because variation in "staining intensity" among individuals is far greater for *Amy-X* than for *Amy-1*. In particular, *Amy-1* activity is almost invariably present, while *Amy-X* activity is often absent, and the probability of its presence is clearly influenced by the treatments described below. Experiment 11 addressed this assumption directly and showed that changes in the intensity of both isozymes do occur, although the changes are greater for *Amy-X*.

Electrophoretic and staining techniques

Freshly sacrificed amphipods were homogenized in 20 to 150 microliters of buffer (0.01 *M* tris-Cl, pH 7.6, 0.001 *M* beta-mercaptoethanol and 0.001 *M* EDTA) and the homogenates were cleared by centrifugation (10,000 × *g*, 10 min). Supernatant was used directly for electrophoresis or for enzyme assays. Samples (10 microliters) were mixed with an equal volume of 40% sucrose in water containing bromphenol blue as a tracking dye, and electrophoresed (PAGE) in polyacrylamide slab gels (7.2%T:2.5%C) using the Ornstein-Davis buffer system (Smith, 1968) for 1.5 to 2.5 hours at 20 to 25 mA. Relative mobility values (RM) were calculated with respect to the migration of tracking dye.

Amylase activity was visualized by incubating the gel on a "starch plate" subsequently developed with a Gram's iodide/iodine solution (2 g KI, 1 g I_2, in 300 ml. H_2O). The "starch plates" were prepared by coating a "gelbond" sheet (Marine Colloids, Inc.) to a thickness of 1 mm with starch solution (1% soluble starch or amylopectin, 1% agarose in 0.02% NaCl, 0.02 *M* tris-HCl pH 7.0) and evaporating it to dryness.

The relative expression of *Amy-1* and *Amy-X* isozymes was scored visually on a scale of 1 to 7. Samples exhibiting activity in only one of the two zones were rated either "1" (*Amy-1*) or "7" (*Amy-X*). Equal expression of both bands was scored "4," just noticeable differences, "3" or "5," while great inequalities, "2" or "6."

This method of scoring the gels was chosen because of the practical difficulties involved in obtaining quantitative activity data from the developed starch plates. The starch coatings were not uniform in thickness among plates, and even on the same plate the intensity of the background iodine staining was variable. Furthermore, the amount of activity for each isozyme varied among animals, so to visualize all of the samples run on a given gel, the starch plates were over-incubated. This procedure brought many of the samples, which surely differed in activity, to the identical end point, achromaticity. For these reasons, the relative activities of bands were taken in most of the experiments as ordinal, rather than rational, variables. In experiment 11, for reasons detailed below, densitometry was employed, although the quantitative data obtained were used only for descriptive purposes.

Statistics

Because the data consisted of ordinal variables, non-parametric statistics were used for all tests: contingency was tested using Chi Squared statistics, or Fisher's exact test when one or more cells had low expected values. The effects of diet and temperature were tested for significance using Wilcoxon Statistics, or the Fisher test, when applicable.

RESULTS

Zones of amylase activity resolved by PAGE

Two principal zones of amylase activity were resolved in all three species by PAGE. The more anodal zone (*Amy-1*) has an RM of about 0.9, while the slower

TABLE I

The effects of diet and temperature on the relative expression of Amy-1 *and* Amy-X*

Replicate number	Time and temperature	Feeding: Control 1	Control 2	Brown	Unfed	Agarose	Amylose Agarose	Amylopectin Agarose	Shrimp	Amylose statistics
1	2D, 22°C 1D, 22°C		2.1 ± 0.4 8		1.4 ± 0.5 8		4.1 ± 2.0 7	3.3 ± 2.0 9		T = 37.5 *P* = .036
2	20D, 22°C 5D, 22°C	1 ± 0 8	1.5 ± 0.6 4	1.5 ± 0.6 4	1 ± 0 5	1 ± 0 5	3.0 ± 0.7 5			T = 16 *P* = .002
3	6D, 6°C 2D, 6°C		1.2 ± 0.5 5		1.1 ± 0.4 7	1.4 ± 0.5 7	3.0 ± 1.3 6	1.2 ± 0.5 5		T = 18.5 *P* = .041
4A	1D, 5°C 3D, 5°C		4.6 ± 1.3 5	4.1 ± 0.8 8	4.2 ± 0.8 5	4.9 ± 0.8 8	4.4 ± 0.9 5	4.4 ± 1.1 5		*P* = NS
4B	Same 4D, 5°C		3.6 ± 0.6 5	3.8 ± 0.5 5	3.4 ± 0.6 5	4.6 ± 1.1 5	5.6 ± 0.6 5	3.8 ± 1.3 5		T = 15 *P* = .008
5A	21D, 14°C 1–2D, 4°C	1 ± 0 5	2.2 ± 0.4 6				3.0 ± 1.3 7			*P* = NS
5B	Same 1–2D, 22°C		1 ± 0 6				2.7 ± 1.6 7			T = 10 *P* = .029
6A	>60D, 22°C 1D, 4°C	2.2 ± 1.1 5	2.0 ± 0 5				2.2 ± 0.5 5			*P* = NS

6B	Same 2D, 4°C		4.3 ± 1.3 4			5.8 ± 0.5 5			P = NS
7A	5D, 10°C 1–6D, 10°C		1.9 ± 0.5 4	1.9 ± 0.6 8				2.1 ± 0.4 7	
7B	Same **		1.2 ± 0.5 4					3.5 ± 1.0 4	
8A	3D, 10°C 7D, 10°C			1.9 ± 2.2 5	1.6 ± 0.7 9	5.3 ± 1.0 10	1.2 ± 0.4 9		
8B	Same ***		1.9 ± 0.3 9			3.9 ± 1.6 4	2.0 ± 0.7 4	2.0 ± 0.2 10	T = 23 P = .006
9	**** 1–2D, 11°C	1.3 ± 0.5 6				2.3 ± 1.0 6	1.1 ± 0.4 8		T = 28 P = .094
10	>60D, 20°C 3D, 10°C						4.3 ± 2.1 7	5.8 ± 1.5 9	

* Mean indices of expression ± one standard deviation are listed on the first line for each replicate, sample sizes on the second line. The first lines in the second column list the holding temperatures and times (in days) prior to the experimental treatments, while the second lines give the durations and temperatures of the experimental treatments. "Control 1" animals were sacrificed at the start of the experiment, "Control 2" animals were maintained on green algae for the duration, and "Unfed" animals were starved for the duration. The other diets are described in the text. All animals were maintained on green algae prior to the experiment, with supplements of shrimp meat in replicates 6, 7, and 9. Wilcoxon T statistics for the comparisons of Amylose fed and Control 2 animals and their associated two-tailed probabilities are listed in the last column.

** Starved 4D, then refed for 1–2D, 10°C.

*** Starved 6D, then refed 1D, 10°C.

**** 4D, then starved 8D, 11°C. T test compares "Amylose" and "Control 1."

zone (*Amy-2*) has an RM of about 0.55. In all cases, whole organism extracts exhibited far more activity in zone 1 than in zone 2. In each zone, extracts of individual animals exhibited either one or two bands. Progeny analyses of sibships are consistent with the hypothesis that double banding of *Amy-2* results from heterozygosity and single banding from homozygosity (R. Borowsky, unpub.). This is generally true for amylase in other animal species (Karn and Malacinski, 1978), true in the isopod *Asellus aquaticus* (Lomholt and Christensen, 1970), and is assumed here to be so for *Amy-1* also. Banding patterns, double *versus* single, were uncorrelated between zones, and the zones, therefore, appear to be coded for by different loci.

The third band (*Amy-X*, RM = .87) is the focus of this paper, and was exhibited weakly by some individuals. The expression of *Amy-X* in field caught animals was generally so poor that there was little likelihood of mistaking an individual with *Amy-X* activity for an *Amy-1* heterozygote.

Amy-X in natural populations

The frequency of field collected *Amy-X* variant individuals differed among species and season of collection. For individuals collected from July through September, the frequencies of *Amy-X* variants were 0 out of 216 in *G. palustris*, 2 out of 105 in *G. mucronatus*, and 19 out of 66 in *G. lawrencianus* (Heterogeneity $\chi^2 = 16.34$, df = 2, $P < .01$, ignoring contributions from the smallest cells; overall $\chi^2 = 85.2$). That is, *Amy-X* was most common in the deeper water species and least common in the species with the highest distribution. For individuals collected during March and April, shortly after these species returned from overwintering in deeper waters, the frequency of *Amy-X* was 24 out of 41 in *G. palustris* and was 4 out of 12 in *G. lawrencianus*. The difference in frequency of *Amy-X* between seasons of collection is highly significant for the *G. palustris* sample ($P < .001$, Fisher's exact test), although not for the *G. lawrencianus* sample. All of these animals had *Amy-1* activity.

The apparent seasonal variation in the occurrence of *Amy-X* in *G. palustris* implies that the *relative amounts* of activity in the *Amy-X* and *Amy-1* zones are controlled by environmental factors and suggests the hypothesis that *Amy-X* is a "deep water" or "cold water" isozyme, while *Amy-1* is a "shallow water" or "warm water" isozyme.

Induction of Amy-X by diet and temperature

In order to determine whether the expression of *Amy-X* could be influenced by environmental conditions, ten experiments were performed in which diet and maintenance temperature were varied. *Gammarus palustris* (n = 347) were used for these experiments, and the relative expression of *Amy-1* and *Amy-X* was scored by visual inspection of the stained starch plates after PAGE. Table I specifies the experimental conditions and lists the results as mean scores ± standard deviations.

The results can be summarized as follows: A diet of amylose-agarose increased expression of *Amy-X* over control values in ten of eleven experimental subsets (expts. 1–6, 8, and 9: sign test, two-tailed $P = .012$). In six instances, the individual differences were significant or highly significant (Table I).

Agarose alone in the diet had no effect on the expression of *Amy-X* (4 cases, expts. 2–4 and 8). Neither did starvation for the brief periods employed (one to seven days, expts. 1–4, 7, and 8), nor brown algae in the diet (expts. 2 and 4). In experiment 7b, shrimp meat significantly increased the expression of *Amy-X* (Fischer's exact two-tailed $P = .029$), but in experiment 8 it had no effect. Although shrimp in the diet is associated with prominent expression of *Amy-X* in experiment 10, this experiment

was inadequately controlled and the effect of shrimp must be considered unresolved. Amylopectin-agarose failed to increase the relative expression of *Amy-X* in five experiments (1, 3, 4, 8, and 9).

Reduction in temperature also appears to induce *Amy-X*. In experiment 5, animals were maintained on green algae for three weeks at 14°C and then shifted either to 4° or 22°C. Half were retained on a diet of green algae and the other half were shifted to amylose-agarose. A control group was sacrificed prior to the changes in conditions. In the set retained on green algae, increase in temperature had no effect on *Amy-X* expression while decrease in temperature increased its expression (4° *vs.* 22°C control 2 groups; Wilcoxon $T_{6,6} = 21$, $P = .0022$). The set shifted to amylose-agarose exhibited increased expression of *Amy-X* regardless of temperature, although those shifted to low temperature exhibited better expression than those shifted to high temperature. In experiment 6, juveniles that had been born and raised in the laboratory on a diet of green algae and shrimp meat at 22°C were switched to 4°C and either green algae or amylose-agarose. A control group was sacrificed at the time the experimentals were shifted and the experimentals were sacrificed after one and two days. No differences in *Amy-X* expression were found in the experimental group one day after the shift, but in both feeding groups *Amy-X* expression was significantly increased by the second day (Amylose 6A *vs.* 6B, $T_{5,5} = 15$, $P = .008$; control 1 *vs.* 6B control 2, $T_{4,5} = 12$, $P = .064$: 6A *vs.* 6B control 2 groups; $T_{4,5} = 10$, $P = .016$). While the increased expression can be attributed to diet in the amylose-agarose group, it must be attributed to the temperature change in the control group.

An eleventh feeding experiment was performed in order to determine the effect of mussel glycogen in the diet. *Gammarus palustris* were collected in the field and transferred to filtered water at 19°C for four days. During that period one group was fed agarose flakes, another was fed amylose-agarose, and the third was fed glycogen-agarose. At the end of the feeding period the animals were frozen whole and lyophilized. Six pooled samples of five animals each were made up for each of the three treatments and the samples were weighed (dry) and ground in buffer at a ratio of 1:20 (W:V). The samples were cleared by centrifugation, and 20 microliters of each were used for electrophoresis. Because of the uniform dilution and the equal sample sizes, semi-quantitive comparisons among bands on the stained plate were possible in this experiment.

The agarose fed animals expressed *Amy-X* poorly, if at all. In contrast, both the amylose and the glycogen fed animals clearly expressed *Amy-X*. Therefore, glycogen feeding also induces the synthesis of *Amy-X*. Of greatest interest, however, is the observation that the overall intensity of *Amy-1* banding in the amylose treatment was uniformly lighter than that in the other treatments (Fig. 1). This shows that amylose treatment effects a true differential induction of *Amy-X* over *Amy-1* instead of simply increasing levels of both isozymes to a point at which the *Amy-X* band can be discerned on the plate.

Is Amy-X *an artifact?*

Takeuchi *et al.* (1975) have shown that the electrophoretic mobility of rat liver amylase depends upon whether the source animal had been well-fed or fasted prior to sacrifice. Amylase from fasted animals had a greater anodal mobility than amylase from well fed animals. This difference proved to be due to glycogen bound to the amylase of well fed rats and the effect was duplicated *in vitro* by mixing amylase from fasted rats with 4% glycogen, prior to electrophoresis. Such a phenomenon

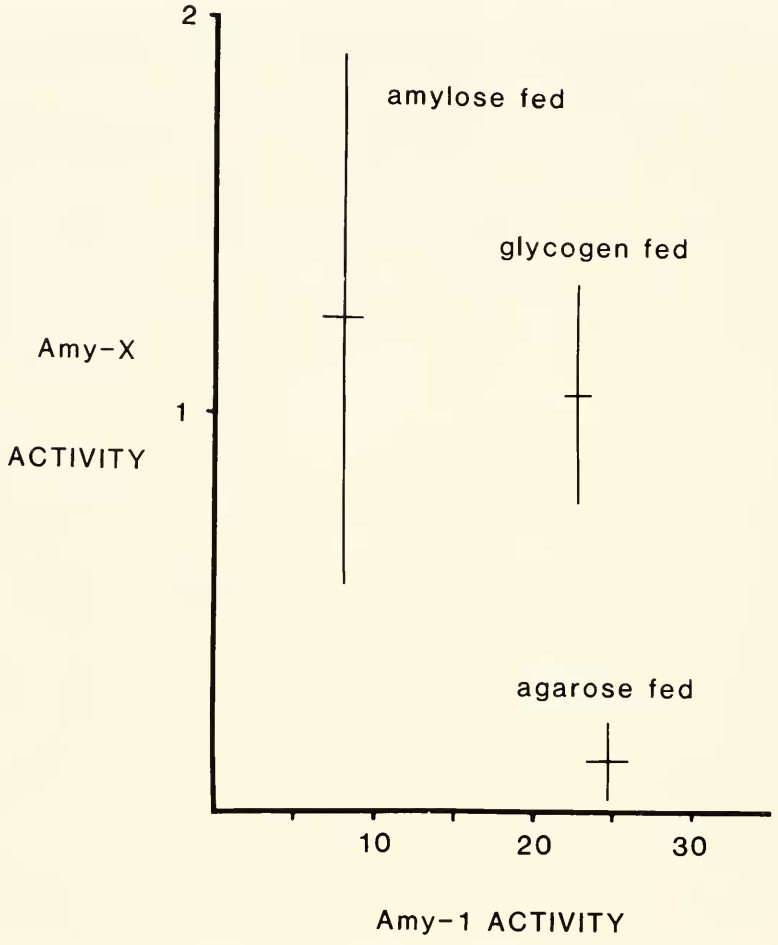

FIGURE 1. Relative activities of *Amy-1* and *Amy-X* depend upon diet in *G. palustris*. Activity is expressed in arbitrary units proportional to the area under each peak obtained by densitometry (Gelman instruments) of the original starch plate used for experiment 11. Each point is the mean of the six samples run (five for amylose) and the bars denote one S.E. of the mean.

might account for the generation of *Amy-X* from *Amy-1* in starch and glycogen fed *Gammarus*, especially since the mobility of *Amy-X* is lower than that of *Amy-1*.

In order to test whether substrate binding could account for the "induction" of *Amy-X*, an experiment was performed in which equal volumes of the same samples were electrophoresed in the presence and the absence of glycogen. Ten microliters of each of five samples from experiment 11 were mixed with equal volumes of water prior to electrophoresis while another set of the same samples were run after mixture with equal volumes of 8% mussel glycogen in water. Glycogen had no effect on electrophoretic mobilities or the relative distribution of activity between *Amy-1* and *Amy-X* in the samples, although the staining intensities of the samples pre-mixed with glycogen were uniformly less intense than those of the samples diluted with water.

Discussion

The data establish three things: first, there are two amylase isozymes of high anodal mobility in *Gammarus,* and, in *Gammarus palustris,* they can be induced differentially. At high temperatures (14°–22°C) and on a diet of green macro-algae and shrimp, *Amy-1* is the predominant fast running isozyme and *Amy-X* is present only in trace quantities; lowering the temperature to 4°C or feeding a diet high in amylose or glycogen causes the differential induction of *Amy-X.* Second, field caught *G. palustris* individuals express *Amy-X* activity with higher frequency in the winter and spring than in the summer and fall. This suggests a seasonal pattern of variation in amylase phenotype. Third, in summer and fall collections of the three species, the expression of *Amy-X* was greater in *G. lawrencianus* than in *G. mucronatus,* and least in *G. palustris.* This suggests an ecological zonal pattern of variation.

The experimental results are consistent with the observations on field caught animals and suggest ways to account for observed variation among seasons and species. The apparent seasonality in expression of amylase observed in *G. palustris,* for example, could be accounted for by seasonal variation in temperature. Low temperatures during the winter would enhance the expression of *Amy-X* phenotype. It is possible, also, that a seasonal variation in diet could be a contributing factor. Gut content analyses show that *G. palustris* feeds year-round (Gable and Croker, 1977), and also that their diet varies seasonally. While the major portion of their diet year-round consists of unidentifiable detritus, during the winter the identifiable fraction contains a greater proportion of non-filamentous green algae and lower proportions of diatoms and blue-green algae than during the spring. Thus, either qualitative or quantitative differences among seasons in the amounts of starches and glycogens available could modify the effects of temperature. If intertidal zone diets differed in general from subtidal diets this might also account for the differences observed among species. Because of their zonation, *G. lawrencianus* is more exposed to deeper water food sources than is *G. mucronatus* which, in turn, has a greater exposure than *G. palustris.*

Differential amylase induction in *Gammarus* might function to adapt the organism to seasonal variation in temperature and the structure of available substrates. Differential isozyme induction has been cited before as a potential mechanism for temperature acclimatization (Hochachka and Somero, 1968; Somero, 1969a), and the concept can be broadened to cover adaptation to variation in any type of environmental factor, including substrate structure. If the enzyme form induced by a given set of environmental conditions has catalytic properties optimizing function under those conditions, the induction would be adaptive, both physiologically and evolutionarily.

A clear example is provided by acetylcholinesterase isozymes in rainbow trout brain tissue (Baldwin and Hochachka, 1970; Baldwin, 1971). One acetylcholinesterase isozyme is induced after acclimatization at high temperatures (17°C), a different form is induced at low temperatures (2°C), and both forms are present at intermediate temperatures. The differential induction appears to be an adaptive response optimizing the efficiency of catalysis over a temperature range, because the isozymes have temperature dependent K_m curves with minima at their induction temperatures. An analogous situation has been reported for pyruvate kinase in the Alaskan King-Crab by Somero (1969a), although in this case the two enzyme forms are apparently coded for by a single locus and are interconvertible. Other examples of temperature-dependent differential enzyme induction in fish and invertebrates are provided by Flowerdew and Crisp (1976), Kent and Hart (1976), and Marcus (1977).

Amy-X is not a simple Mendelian allelic variant of *Amy-1* and its presence is clearly inducible. While it is possible that *Amy-X* and *Amy-1* are coded for by different loci, this is by no means certain. Evidence is accumulating that protein diversity can result from variable processing of RNA, or post-translational modification of polypeptides. Furthermore, such modifications have been documented for amylase systems. Kikkawa (1964), for example, has shown that each major amylase band from *Drosophila melanogaster* is associated with a minor band, and both are coded for by the same allele. MacDonald *et al.* (1977) have demonstrated that amylase mRNA from dog pancreatic tissue is translated *in vitro* into a polypeptide 1500 Daltons larger than the native enzyme. This suggests post-translational modification of the protein. In mice, amylase mRNA from both liver and salivary tissue exhibits a heterogeneity caused by variable processing (Hagenbuchle *et al.*, 1981). Craik *et al.* (1983) have proposed that variable excision of introns from genes such as amylase could account for polypeptide diversity. It is reasonable to suppose that environmental conditions could affect the splice position, and result in the observation of "inducibility." Whether or not *Amy-X* and *Amy-1* are coded for by the same locus, the observations reported here are of importance because they link the phenomenon of differential inducibility to specific environmental factors that are of relevance in nature.

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